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Calprotectin Pegylation Enhanced Its Physical and Structural Properties

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Abstract Calprotectin is member of the S-100 protein family with a wide plethora of intra-and extracellular functions. Anticancer activities, antimicrobial effects and being a qualified disease marker are among the compelling features of this protein to be used as a pharmaceutical agent. However, there are several impediments to applications of protein pharmaceuticals including: proteolytic degradation, short circulating half-life, low solubility and immunogenicity. Pegylation is a common bioconjugation polymer capable of overcoming these drawbacks. Recombinant expression and purification of calprotectin along with its pegylation would result in enhanced pharmacodynamic and pharmacokinetic properties. Our florescence spectroscopy and far Ultraviolet-optical density results indicate that pegylation altered the physical and structural properties of the calprotectin to become in a more stable and functionally active state. Due to enhanced pharmacodynamic and pharmacokinetic properties of the calprotectin via pegylation, this study would pave the way for better in vitro and in vivo validations of calprotectin applications in medical practice.

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Abbreviations

CD	Circular dichroism
IPTG	Isopropyl-thio-β-D-galactoside
mPEG	Methoxy poly ethylene glychol
MRP	Myeloid inhibitory factor related protein
N_{i2}+-NTA	Nickel-nitrilotriacetic acid
PEG	Polyethylene glycol
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

1 Introduction

Calprotectin is a calcium- and zinc binding protein of the S-100 protein family with a wide plethora of intra- and extracellular functions. It plays a pivotal role in the regulation of inflammatory processes and immune response. The protein complex is a 24 kDa heterodimer, incorporating a light (MRP8 also known as RP8/S100A8/L1L/P8) and a heavy (MRP14 MRP14 also known as S100A9/P14/L1H) chain. Calprotectin reported to have antimicrobial activities towards bacteria and fungi. The antimicrobial activity most likely is exerted via the ability of calprotectin to chelate Zn^{2+} which is essential for microbial growth. Moreover, it can induce cell death via autophagy and apoptosis; it have protective role in preventing exaggerated tissue damages; it acts as a potent amplifier of inflammation in autoimmunity as well as in cancer development and tumor spread, while regulates cell survival, neutrophil number and apoptosis [1–4].

The utilization of proteins and peptides as medicinal substances become to be an inevitable part of contemporary

biological studies. Despite of the unprecedented application of biopharmaceuticals in prevailing medical practice, there are still several impediments to their applications including: proteolytic degradation, short circulating half-life, low solubility and immunogenicity. Therefore, understanding their mechanism of action and better efficiency necessitates additional improvements. In this regard, the covalent derivatization of biomolecules or "Bioconjugation" is a novel approach, garnering a lot of attention. Bioconjugated molecules could be expeditiously employed for discovery of biological interactions, rigorous biochemical analyses, diagnosis, in vivo imaging and industrial applications [5]. In this regard, there have been a staggering progress in molecular altering technologies and formulation, enabling proteins and peptides to be delivered in vivo with enhanced pharmacodynamic and pharmacokinetic properties [6–8]. One of the well-established techniques in the field of bioconjugation is the conjugation of polyethyleneglycol (PEG) molecules to proteins (commonly referred as PEGylation). PEG is biologically inert, nontoxic, nonimmnogenic polymer. The predominant use of protein PEGylation has been associated with decreasing the immunogenicity and increasing the biological half-life of protein drugs [9]. Creating a reactive functional group is the first step for PEGylation of molecules. A wide variety of commercially available chemistries have been developed for protein PEGylation [10], most of which focused on different ways to synthesize PEG derivatives capable of conjugating with free amino groups to form amide bonds. This method is preferred for most applications due to the abundance of free amino groups in proteins (Lysine and Arginine residues) and the stability of the formed amide bonds [11]. Using cyanuric chloride as a coupling agent is one of the original methods of protein derivatization with PEG molecules [12].

All of the aforementioned properties make calprotectin a compelling therapeutic target for clinical applications. Therefore, using a bioconjugation method like PEGylation technique seems intriguing to decrease its proteolytic degradation, increase its short circulating half-life and deal with its solubility and immunogenicity. In present study PEG conjugation technique was employed to PEGylate an s100A8/9 complex, produced by recombinant expression and purification. Assessing the physical and structural properties of the PEGylated complex indicates the acquisition of a more stable and functional state for the final complex.

2 Materials and Methods

2.1 Recombinant Vectors Harboring S100A8/A9

According to standard protocol [13], S100A8 and S100A9 sequences (ordered from Biomatik company) were subcloned into pET15b vector [Novagen (EMD Millipore)] and then transformed into *E.coli* BL21 (DE3) competent cells, grown on ampicillin (100 μg/l) containing LB agar plates. The sequences of the study are used from a human source were previously employed in our lab in conduction other studies [14, 15]. One of the transformed white colonies for both S100A8 & S100A9 were selected for confirmation by sequencing (using T7 universal primers) and expression of recombinant protein.

2.2 Recombinant Expression of S100A8 and S100A

Transformed colonies were cultured in LB broth containing ampicillin (100 μg/l) with concurrent shaking. Induction of recombinant subunits (r-S100A8 and r-S100A) was done at OD600 of 0.6 using 1 mM IPTG for 4 h at 37 °C. Following the induction, cells were collected by centrifugation at 4200 g for 15 min at 4 °C and lysed employing sonication (the conditions were as follow: three cycles of 20 s sonication, 30 % power and on ice), while suspended in PBS lysis buffer (NaH_2PO_4 1.9 mM, Na_2HPO_4 8.1 mM, NaCl 154 mM, pH = 7.2) on ice. The sonicated precipitate was centrifuged at 12000*g* for 30 min at 4 °C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 % polyacrylamide gels according to Laemmli protocol [16]. Fifteen μl of protein solution and standard molecular mass marker were mixed with SDS-PAGE sample buffer and heated in boiling water for 5 min. Electrophoresis was carried out at a constant current of 30 mA. The gel was then stained with Coomassie Brilliant Blue R-250 (Sigma) and incubated in de-staining solution with gentle shaking to remove background to visualize protein bands.

2.3 Protein Purification

The cleared supernatant subjected to purification step based on an affinity binding between His6-Tag sequence on the recombinant S100A8 and S100A9 and Ni-NTA resins in the column. The supernatant was loaded on to Ni-NTA column and finally eluted with imidazole solutions from 10 mM up to 150 mM imidazole concentrations (Tris 100 mM, NaCl 25 mM, imidazole 10 mM up to 150 mM, pH = 7.5). Eventually, imidazole was removed from purified protein by dialysis. Dialysis was done by PBS dialysis buffer (NaCl 25 mM, NaH_2PO_4 100 mM, pH = 6.5) for 8 h at 4 °C and stored at −80 °C for further analysis and SDS-PAGE.

2.4 Protein Assay and Complex Formation of r-S100A8 and r-S100A9

The protein concentration was measured by Bradford method [17] using Nano Drop spectrophotometer with

bovine serum albumin as standard. To prepare r-S100A8/A9 complex, equal volumes of r-S100A8 and r-S100A9 (1 μM each), were incubated with calcium chloride (1 mM) in PBS dialysis buffer for at least 15 min at 25 °C, as previously described [18]. The complex formation was investigated by fluorescence and CD spectroscopy.

2.5 Activation of mPEG33KD with Cyanoric Chloride

The methoxy-PEG (mPEG) (5gr), 151 μM, was dried in a vacuum desiccator for 1.5 h at 80 °C, then dissolved in 40 ml anhydrous benzene and stirred for 10 min at 70 °C. Then, a combination of cyanoric chloride (33.1gr) and anhydrouse sodium carbonate was added after 30 min. The solution stirred for 24 h at room temperature and then filtered. Thereafter, for three cycles 200 ml petroleum ether was added gradually (solution A) and the precipitate was dried out. The precipitate was dried in vacuum desiccator and ultra-filtered by filter paper (solution B) to achieve activated mPEG with cyanoric chloride (monomethyl ether, poly ethylenglychol 4, 6 dicholoro1, 3, 5 triazin). The produced white powder stored in −20 °C.

2.6 Coupling of mPEG-triazin and Recombinant Protein Complex

Two hundred μl of S100A8/9+Ca complex (1.9 mg/l), was dissolved in 100 ml of 0.1 M sodium tetraborate at 3 different pH (6.5, 7.2 and 9.2). This solution and activated mPEG in different ratio concentration (0.01, 0.025, 0.001 mM) were mixed at 4 °C and room temperature. After 1 h uncoupled mPEG was removed by dialysis using PBS dialysis buffer (Nacl 25 mM, NaH_2PO_4 100 mM, pH = 7.2) for 18 h according to [12].

2.7 Circular Dichroism (CD) Spectra

Recombinant S100A8/9 (r-S100A8/9) complex and PEGylated r-S100A8/9 (mpeg- r-S100A8/9) complex were used to assess the effects of PEGylation on structure and proteolytic stability of these complexes, using CD spectra and trypsin treatment. The data were recorded in dialysis buffer (pH = 6.5) at concentration of 0.05 mg/ml and reads were performed with 1 mm path length quartz cell at 25 °C. All spectra were collected from 190 to 260 nm and the background was corrected using the buffer as blank. The r-S100A8/9 complex was incubated with mPEG (0.025 mM at pH = 6.5, 7.2 and 9.2) for 1 h. To analyze the proteolytic consequence of trypsin treatment on S100A8/9 complex in presence and absence of mPEG trypsin (0.01 %) was incubated with the complexes (with 1:1, 1:3 and 3:1 ratios, at pH = 7.2, for a time range includes 10, 30 and 60 min, at room temperature). The data were expressed as molar elipticity (deg.cm2/dmol) considering a mean residue number of 207 and average molecular weight of 25 kDa for r-S100A8/A9 complex [19]. The molar ellipticity was determined as $[\theta] = [100 \times (MRW) \times \theta obs/(cl)$, where θobs is the observed ellipticity in degrees at a given wavelength, c is the length of the light path in cm [20].

2.8 Intrinsic Fluorescence Spectroscopy

The fluorescence measurements of r-S100A8/A9 complex, following a treatment with calcium chloride (1 mM), and PEGylated samples (0.001, 0.025 and 0.01 *m*M) in 50 mM PBS buffer were performed using a Cary 100 biospectrofluorimeter equipped with a 150 W xenon lamp, a DR-3 data recorder and emission slits set at 5 and 10 nm [21]. Intrinsic fluorescence using cuvettes with a 1 cm path length and with excitation wavelength of 295 nm was used in all of these studies.

3 Results

3.1 Gene Sub-cloning

The sub-cloning of the genes was successfully confirmed by sequencing of the resulted vectors using T7 universal primers.

3.2 Protein Expression, Purification and PEGylation

As depicted in Fig. 1, the protein expression of S100A8 and S100A9 resulted in overexpression of both subunits

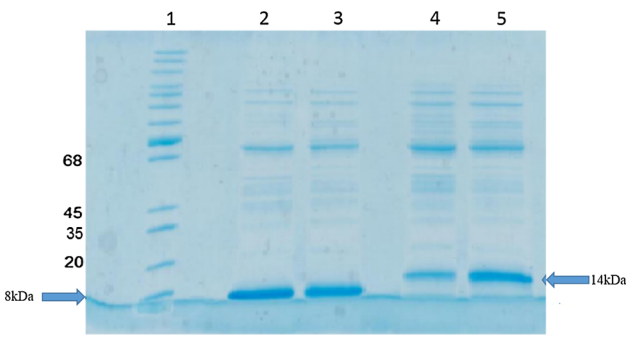

Fig. 1 The SDS-PAGE analysis of recombinant S100A8 and S100A9 expression (*1* Marker, *2* S100A8 expression (lysed bacteria), *3* S100A8 expression (the supernatant of the lysed bacteria after centrifugation) *4* S100A9 expression (the supernatant of the lysed bacteria after centrifugation) and *5* S100A9 expression (lysed bacteria))

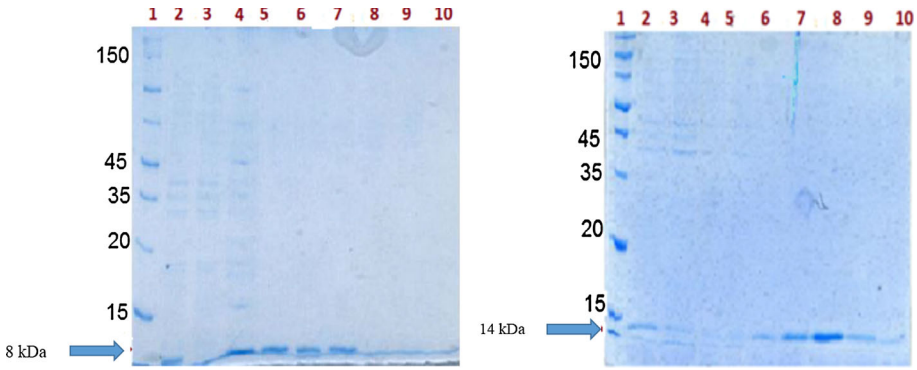

Fig. 2 The SDS-PAGE analysis of purification attempts at various concentrations of imidazole. The picture on the *left* belongs to S100A8 and the picture on the *right* belongs to S100A9 (*1* marker, *2* supernatant, *3* flow through, *4* 50 mM, *5* 75 mM, *6* 100, 7.150 mM, *8* 200 mM)

migrating with an apparent molecular weight of about 8 and 14 kDa compared to the logarithm of molecular weight of standard marker proteins. The protein assay results indicate that the expression for S100A8 and S100A9 are equal to 0.1 and 0.07 mgr/mL respectively. Moreover, according to the electrophoresis, fractions of 100 and 150 mM concentrations of imidazole appeared to have single and the broadest band for S100A8 and S100A9 respectively (Fig. 2). Therefore, these concentrations were selected for protein purification. Figure 3 shows the quality of recombinant protein preparation following dialysis experiments. The PEGylation of the r-S100A8/9 was successfully attempted as the next step.

3.3 Fluorescence and Far UV-CD Studies of r-S100A8/A9 Complex

The assembly of r-S100A8 and r-S100A9 subunits to the quaternary structure of r-S100A8/9 leads to structural changes. The results of fluorescence and far UV-CD showed that increasing the concentration of mPEG clearly enhanced the intensity of r-S100A8/9 complex. The spectroscopic properties of mPEG- r-S100A8/9 complex with different concentrations of mPEG are shown in Fig. 4. The fluorescence and far UV-CD spectra indicate significant diversity between r-S100A8/9 complex and mPEG-r-S100A8/9 complex in different concentration of mPEG. Figure 5 shows the effect of pH change on the bioconjugation yield using fluorescence and far UV-CD analyses. Interestingly, significant differences were observed

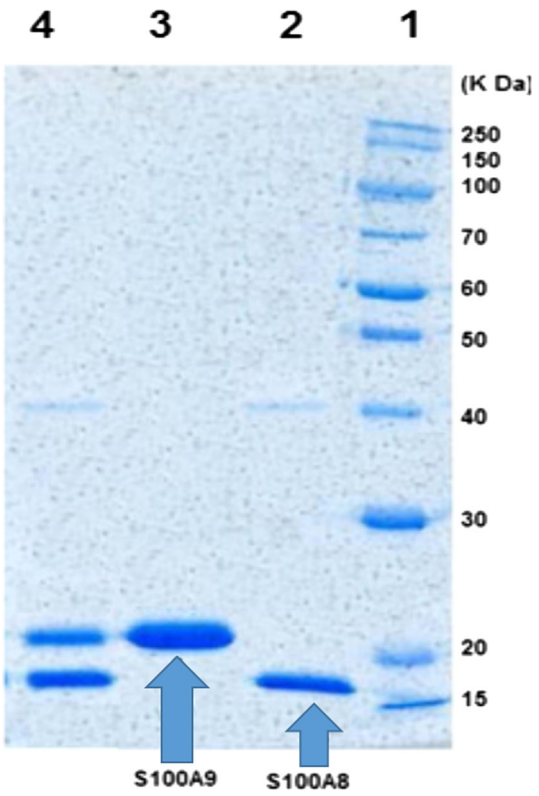

Fig. 3 The SDS-PAGE analysis for dialyzed S100A8 and S100A9 proteins (*1* marker, *2* S100A8 expression, *3*. S100A9 expression and *4*. Both proteins together)

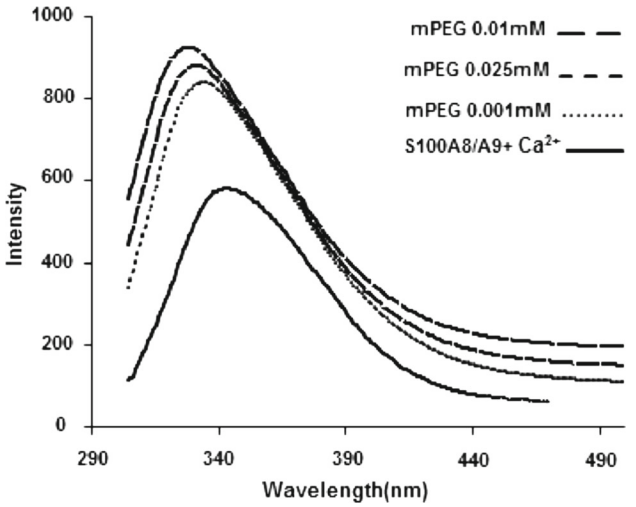

Fig. 4 The spectroscopic properties of mPEG- r-S100A8/9 complex with different concentrations of mPEG. *From top to bottom the curves* belong to florescence emission of S100A8/9 complex PEGylated in 0.01 mM mPEG, 0.025 mM mPEG, 0.001 mM mPEG and without mPEG

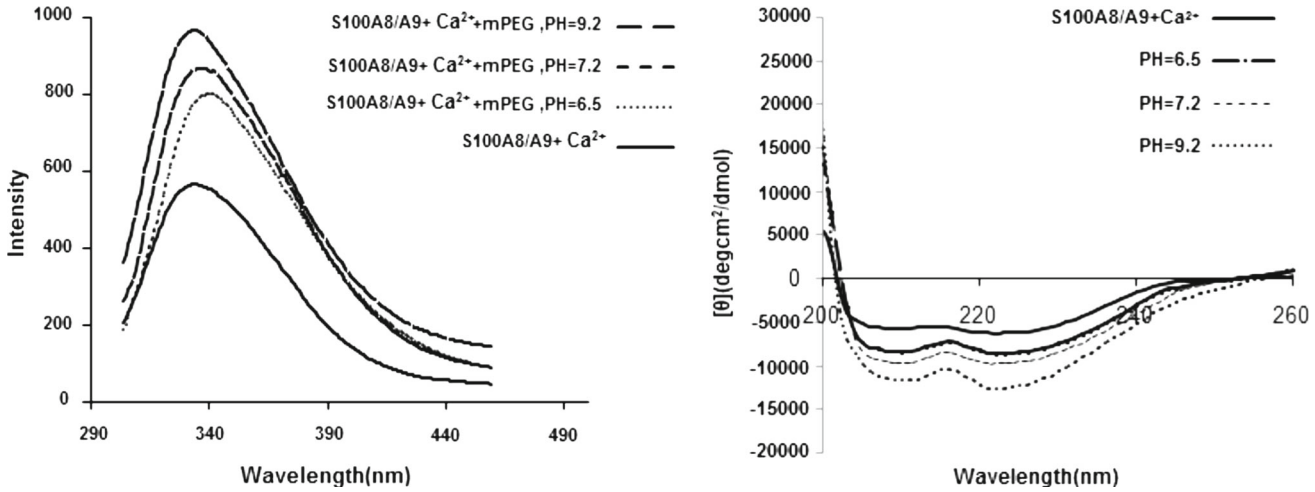

Fig. 5 Fluorescence (*left*) and far UV-CD (*right*) analyses to assess pH effects on PEGylation yields. From *top to bottom* the fluorescence curves belong to PEGylated r-S100A8/9 complex in pH = 9.2, PEGylated complex in pH = 7.2, PEGylated complex in pH = 6.5 and nonPEGylate complex in PH = 6.5, while this order is vice versa in UV-CD curves

between pH 9.2, 7.2 and 6.5, where the protein is fairly stable. As results obtaining from fluorescence and far UV-CD analysis indicate (Fig. 6), there is an apparent difference between PEGylated protein and native form responding to the trypsin treatment in varying concentrations and duration. Table 1 lists the percentages of alpha helixes and beta sheets of the r-S100A8/9 complex in various conditions. Our results indicate that the PEGylation leads to increased alpha helix and beta sheet content of the complex.

4 Discussion

The ongoing studies on calprotectin protein elucidated significant aspects of its biology. Anticancer activities, antimicrobial effects and being a qualified disease marker are among the compelling features of this protein [19, 22–24]. However, conduction of structural and functional studies as well as investigating molecular bases of protein–protein and protein–ligand interactions would not be feasible without an abundant source of expressed S100A8/9 complex. Recombinant expression of calprotectin is previously reported by several research groups [25, 26]. Since Ca^{2+} is essential to form a r-S100A8/9 complex, a sufficient source of Ca^{2+} should be added to have functional heterodimers of calprotectin [27, 28]. The heterodimer assembly is energetically favorable due to burying surface accessible regions of both S100A8 and S100A9 proteins. Residues spanning 1–42 and 51–78 within S100A9 and residues spanning 4-45 and 59-86 within the S100A8 are actively involved in heterodimer assembly. A unique salt bridge between Asp30 from S100A9 and His87 and His83 from S100A8 is the main stabilizing bond [29]. Displacement of aromatic residues from the more hydrophobic patches of the protein onto the surface of the complex, following the heterodimer assembly, is in line with the Ca^{2+} tendency to increase the surface hydrophobicity of the proteins and protein assembly [20].

The FDA approved the PEG molecule to be safe for clinical uses with molecular weights of over 1000 Da [9, 30]. There are several properties for PEG molecule rendering this molecule as an amenable agent to improve the desired protein features. These properties include: 1- lack of toxicity and high mobility, 2- lack of immunogenicity and allergenicity, 3- flexibility and high solubility inwater and organic solvents and 4- high hydrophilicity [30, 31]. Due to the high mobility and hydration shell of the polymer chains, PEGylation increases the size of protein complexes considerably more than the molecular weight increase; this leads to slower clearance from the blood stream and lower immunogenicity, which in turn leads to longer biological half-lives for PEGylated drugs. Lower doses needed for effective treatment and thus reduced systemic toxicity are the consequences of achieved longer biological half-lives. Furthermore, PEG has been used to modify water-soluble proteins to solubilize them in organic solvents [9, 30, 32]. Since, protein PEGylation is endowed with bringing about valuable properties, calprotectin PEGylation seems to be a rational method to improve its pharmaceutical properties.

As Abuchowski et al. [12] have pointed out in their study even PEG1900-albumin and PEG-5000-albumin do not enter 4 % acrylamide gel duo to the their high MW. Our PEG/protein complex is much bigger and as expected the complex did not enter the SDS page gel. It should be noted that in the case of PEG activation failure or protein sedimentation, the changes in protein native structure or its

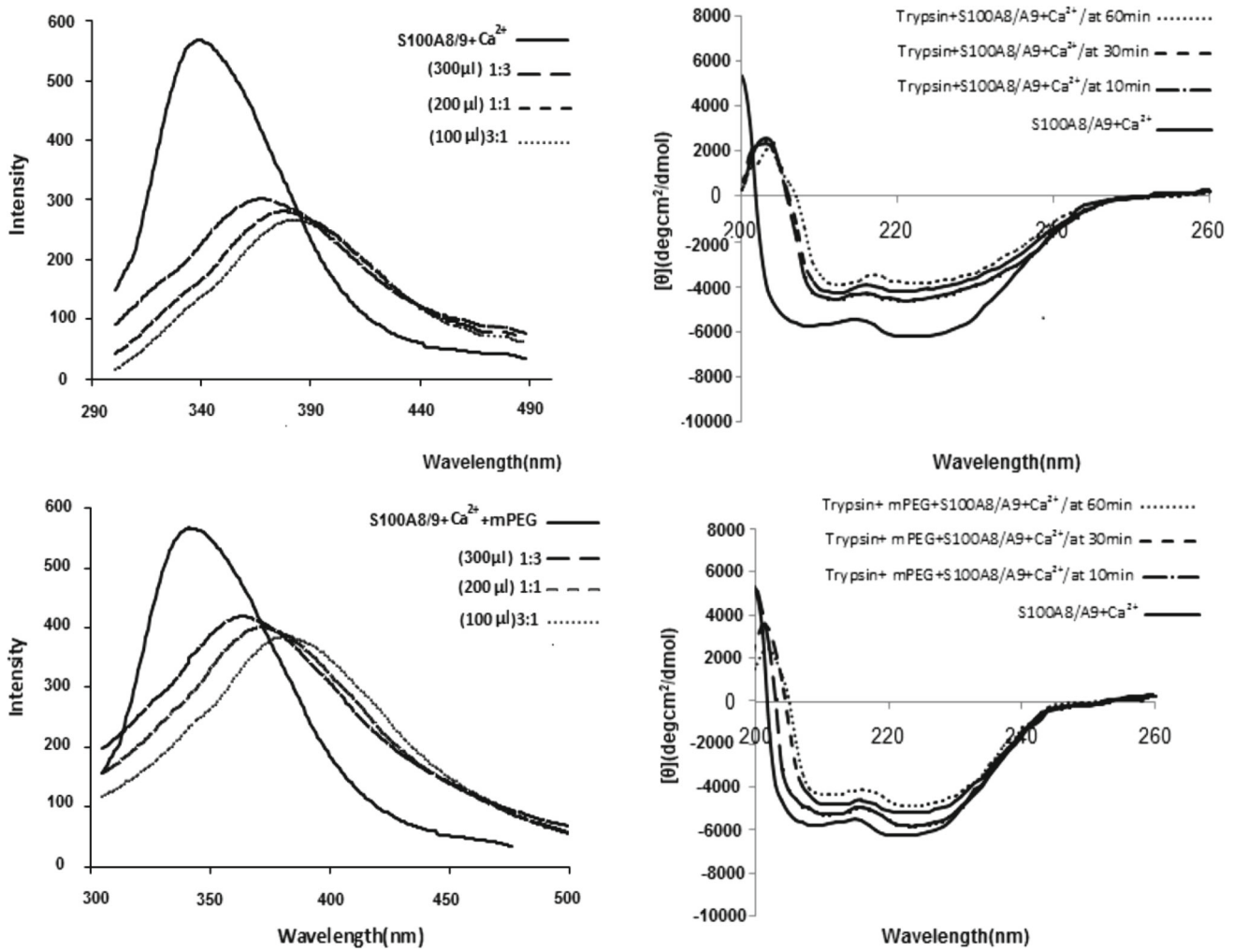

Fig. 6 Fluorescence (plots on the *left*) and far UV-CD (plots on the *right*) analyses to assess trypsin effects on nonPEGylated (plots on the *top*) and PEGylated (plots on the *bottom*) complexes by different concentrations (plots on the *left*) and durations (plots on the *right*. From *top to bottom* the fluorescence curves belong to r-S100A8/9 complex treated with no trypsin, 1:3, 1:1 and 3:1 volume ratio of trypsin/PEGylated complexes. From top to bottom the UV-CD curves belong to r-S100A8/9 complex treated with trypsin for 60 min, trypsin for 30 min, trypsin for 10 min and no trypsin for both PEGylate and nonPEGylated complexes

Table 1 The structural composition of r-S100A8/A9 in different conditions (numbers are in percent of whole protein and all the reactions are in presence of Ca^{2+})

Proteins	α-Helix (%)	β-Sheet (%)	Other structures (%)
S100A8/A9 + PEG PH = 6.5	41.4	21.1	37.5
S100A8/A9 + PEG PH = 7.2	44.6	18.2	37.2
S100A8/A9 + PEG PH = 9.2	52.8	17.3	29.9
S100A8/A9 + PEG + Trypsin0.01 % 10 min	35.8	22.5	41.7
S100A8/A9 + PEG + Trypsin0.01 % 30 min	32.4	23.2	44.4
S100A8/A9 + PEG + Trypsin0.01 % 60 min	31.8	23.7	44.5
S100A8/A9 + Trypsin0.01 % 10 min	31.2	21.9	46.9
S100A8/A9 + Trypsin0.01 % 30 min	27.8	21.1	51.1
S100A8/A9 + Trypsin0.01 % 60 min	27.1	20.6	52.3

degradation would most likely occur. Our results indicated an unchanged pattern of structural features, while the PH was changing. On the other hands, significant changes have been observed changing from the PH of 6.5 to the pH of 9 which is the consequence of behavioral changes of amino acids like lysine in interaction with activated PEG. These

changes are well documented in several studies [33–35]. Moreover, the presence of cyanoric chloride in the solution A (yellow) and its absence in the solution B (blue) apparently indicates chloride binding to PEG molecules and its activation. The PEG and r-S100A8/9 complex interactions are highly pH dependent [36]. Our results confirmed that increasing the pH, changes the amino acids behavior, leading to an increase in florescence emission and a shift into left in its diagram as a consequence of more amenable PEGylation. This would be rooted in the change of the histidine residue behavior at pH $\geq$ 7 and the preliminary role played by the epsilon-amino group of lysines at pH = 9.2. Moreover, the N terminal alpha amino groups of amino acids which are less active in comparison to lysine establish more stable interactions with PEG monomers by reaching more basic pH [37].

It has been demonstrated that the relative amount of alpha helixes and beta sheets of proteins is physiologically significant and this relative proportion could be changed in the case of some disease [20, 38–40]. This kind of structural changes and aromatic residues movement onto the surface of the complex are crucial implementing some of the calprotectin protein functions including: inhibition of the casein function and apoptosis induction [20, 41, 42]. Our far UV-CD analysis indicates that the subunits of calprotectin have a high content of alpha helixes [20]. Although, the presence of Ca^{2+} decreases the alpha helix content, the overall Ca^{2+} changes are negligible. However, as revealed by our analysis pH changes have more serious impact on the content of alpha helixes, could be important exerting various functions of calprotectin. Since, the calprotectin is a member of S-100 protein family, known for the pivotal roles of abundant alpha helixes in their structure and function, PEGylation originated increase in alpha helixes would help their structural integrity and stability. The maximum lambda of the florescence emission plot depends on the pH and the concentration of mPEG. In this regard, our results indicate that increasing the pH and the concentration of mPEG would lead to a decrease in maximum of the wave length or the blue shift.

Trypsin is a commonly used endopeptidase (a serine protease) capable of cleaving the proteins into polypeptides. Use of trypsin to analyze its proteolytic activity on deferent proteins is wide spread. Lysine and arginine residues are among the hot spots of trypsin function. Calprotectin contains numerous lysine residues some of which are located in surface accessible regions. The PEGylation related interactions mainly occur at locations of lysine residues. Preoccupation of these lysine residues by PEG molecules restricts trypsin access to the protein, therefore inhibits the trypsin function in different concentration and time periods of treatment. It could be apparently deduced that the calprotectin PEGylation increases the protein stability and half-life masking its surface in protease tensions. Given the maximum lambda of the florescence emission plot which is a direct consequence of the trypsin activity, it could be deduced that the increase in trypsin concentration would lead to an increase in maximum of the wave length or the red shift.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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